Screening and partial characterization of bacteriocin from lactic acid bacteria in fish gastrointestinal tract

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Abstract

Screening and characterization of bacteriocin from lactic acid bacteria (LAB) in fish gastrointestinal tract were conducted. Four hundred isolates of LAB were screened from gastrointestinal tract of 6 fish species; *Lates calcarifer* (seabass), *Channa striata* (striped snake-head fish), *Clarias batrachus* (catfish), *Orecochromis niloticus* (nile tilapia), *Barbonymus gonionotus* (javanese barb) and *Pangasianodon hypophthalmus* (striped catfish). One effective isolate from seabass was able to produce bacteriocin and designated Sb2. Antibacterial activity test of cell free supernatant (CFS, pH 6.0) was performed against 17 bacteria target strains. CFS of Sb2 displayed growth inhibition of both spoilage and pathogenic bacteria. The maximum bacteriocin activity of 12,800 Au/ml against *Lb. sakei* subsp. *sakei* JCM 1157^T was obtained at the optimum cultivation condition of 30°C for 18 hr. Its bacteriocin activity was partially inhibited by proteolytic enzyme of trypsin, α-chymotrypsin and proteinase K for 50%, 75% and 96.87%, respectively. It was stable at high temperature up to 100°C for 30 min. However, its activity was reduced to 50% (from 12,800 to 6,400 Au/ml) when it was incubated at 100°C for 60 min and 4°C for 7 days. As a result of this, bacteriocin produced from Sb2 could be used as biopreservative.

บทคัดย่อ

การคัดแยกและศึกษาคุณสมบัติของสารแบคเทอริโอซินที่สร้างโดยแบคทีเรียกรดแลกติกจากระบบทางเดินอาหารของปลาโดยทำการคัดแยกเชื้อแบคทีเรียกรดแลกติกจำนวน 400 ไอโซเลทจากปลาจำนวน 6 ชนิดได้แก่ *Lates calcarifer* (seabass), *Channa striata* (striped snake-head fish), *Clarias batrachus* (catfish), *Orecochromis niloticus* (nile tilapia), *Barbonymus gonionotus* (javanese barb) และ *Pangasianodon hypophthalmus* (striped catfish) ซึ่งแบคทีเรียกรดแลกติกไอโซเลท Sb2 เป็นแบคทีเรียที่คัดแยกได้จากระบบทางเดินอาหารของปลากะพงมีคุณสมบัติในการสร้างสารแบคเทอริโอซิน ซึ่งค่ากิจกรรมของสารแบคเทอริโอซินสามารถทดสอบได้โดยนำเฉพาะส่วนใสของอาหารเลี้ยงเชื้อ

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ปรับค่าความเป็นกรดและด่างเท่ากับ 6 จากนั้นนำมาทดสอบกับเชื้อแบคทีเรียเป้าหมายจำนวน 17 ชนิด ผลปรากฏว่า แบคเทอริโอซินที่ผลิตโดยไอโซเลท Sb2 สามารถยับยั้งทั้งเชื้อที่ทำให้เนื้อเน่าเสียและเชื้อก่อโรคได้ โดยสามารถยับยั้งแบคทีเรีย *Lb. sakei* subsp. *sakei* JCM 1157^T ได้มากที่สุด ซึ่งมีค่ากิจกรรมการยับยั้งเท่ากับ 12,800 AU/ml ไอโซเลท Sb2 สามารถสร้างสารแบคเทอริโอซินมากที่สุด เมื่อบ่มเชื้อที่อุณหภูมิ 30°C เป็นเวลา 18 ชั่วโมง (12,800 AU/ml) แบคเทอริโอซินดังกล่าวถูกทำลายด้วยเอนไซม์ trypsin, α–chymotrypsin และ proteinase K โดยคิดเป็นเปอร์เซ็นต์การถูกทำลายเท่ากับ 50, 75 และ 96.87% ตามลำดับ และสามารถทนความร้อนสูงสุดที่อุณหภูมิ 100 องศาเซลเซียส เป็นเวลา 30 นาที เมื่อเพิ่มเวลาปรากฏว่าค่ากิจกรรมลดลงเหลือ 50% และเมื่อนำแบคเทอริโอซินไปบ่มที่อุณหภูมิ 4°C เป็นเวลา 7 วัน ปรากฏว่าค่ากิจกรรมลดลงเหลือ 50%

คำสำคัญ: แบคทีเรียกรดแลกติก แบคเทอริโอซิน ระบบทางเดินอาหารปลา

Keywords: lactic acid bacteria, bacteriocin, gastrointestinal tract, fish

Introduction

Lactic acid bacteria (LAB) are the natural microflora of many fermented food. They serve as preservative because they produce various antimicrobial compound such as organic acid, hydrogen peroxide, diacetyl (Stiles and Holzapfel, 1997; Messens and De Vuyst, 2002), antifungal compounds such as fatty acid (Corsetti et al., 1996) and bacteriocin (Klaenhammer, 1993). Among the antimicrobial substances, bacteriocins have demonstrated great potential as food preservative (Nes and Johnborg, 2004). Bacteriocins are antibacterial proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties (Abee, 1995). They can kill or inhibit the growth of other bacteria (Cleveland et al., 2001).

LAB have become a major source of concern for aquaculture in recent decades (Michel et al., 2007). These organisms are found on several fresh fish species, fish products, or in the intestinal content of fish (Ringø and Gatesoup, 1998). Campos et al. (2006) isolated three bacteriocin-producing LAB from the muscle of turbot (*Psetta maxima*). All strains were identified as *Lactococcus lactis* subsp. *lactis* USC-39, *Enterococcus faecium* USC-46 and *E. mundtii* USC-51. Bacteriocin produced by three LAB strains was able to inhibit *Listeria monocytogenes* and *Staphylococcus aureus*. The aims of this study were screening and characterization of bacteriocin produced by LAB isolated from gastrointestinal tract of various fish in order to use this bacteriocin as biopreservative in food industries in the future.

Materials and Methods

1. Isolation of LAB from fish gastrointestinal tract

LAB were isolated from 6 fish species; *Lates calcarifer* (seabass), *Channa striata* (striped snake-head fish), *Clarias batrachus* (catfish), *Orecochromis niloticus* (nile tilapia), *Barbonymus gonionotus* (javanese barb) and *Pangasianodon hypophthalmus* (striped catfish). Ten grams of gastrointestinal content were homogenized in 90 ml peptone water (Merck, Germany) and 10 folds serially diluted. Subsequently, 0.1 ml of each dilution was spreaded on MRS agar (de Man-Rogosa-Sharpe; Merck, Germany) containing 0.5% $CaCO_3$ (Scharlau Chemie S.A., Spain) and 1% NaCl (Prolabo, Belgium) and then incubated under anaerobic condition at 37°C for 48 h. Only clear zone producing-colonies were secondly selected and stored in 30% glycerol at -80°C for further study.

2. Screening of LAB strains for antibacterial activities

Screening of four hundred isolates for bacteriocin was carried out by using spot-on-lawn method (Ennahar et al., 1999). Cell-free supernatant (CFS) of bacteriocin-producing strains was obtained by centrifugation at 4,000 x g for 20 min and then adjusted to pH 6.0 by applying NaOH (to exclude the effect of organic acid) before sterilization by filter (0.2 µm, Pall, U.S.A). The two layers of agar plate were prepared and 5 ml of soft agar (0.8-1% agar) was added to make top layer which seeded with 10 µl of freshly grown bacterial strain (about 10^7 CFU/ml). Antibacterial activity was tested by spotting 10 µl of CFS onto the top surface of agar plate. Inhibition zone was observed, after overnight incubation at proper temperature as shown in Table 1. The spectrum of CFS was expressed in an arbitrary unit (AU/ml). AU is calculated as (1000/10)D when D is the dilution factor (Parente et al., 1995).

3. Optimum temperature and incubation time for maximum bacteriocin production

The trial of incubation time on growth and bacteriocin production from LAB was performed at 30, 37 and 42°C for 24 h, each 10 ml of sample was collected from the culture every 2 h. Total plate count (CFU/ml), absorbance of bacterial turbidity at optical density (OD) of 600 nm and antibacterial activity (AU/ml) against *Lb. sakei* subsp. *sakei* JCM1157 ^{T} were examined.

4. Effect of proteolytic enzyme on antibacterial activity of CFS

The CFS with final concentration of 1 mg/ml of proteolytic enzyme, trypsin (Sigma, U.S.A), alpha-chymotrypsin (Sigma, U.S.A) and proteinase K (Sigma, U.S.A) at pH 7.0 was digested. Untreated sample without enzyme was used as control. All samples were sterilized by filtering through filter membrane (0.2 µm, Pall, U.S.A) and incubated at 37°C for 3 h. Subsequently, enzyme activity was terminated by heating at 100°C for 5 min. The residual bacteriocin activity was determined by applying spot-on-lawn method against *Lb. sakei* subsp. *sakei* JCM 1157 ^{T} (Ennahar et al., 1999)

5. The effect of heat and chill temperature on bacteriocin activity

The study of heat stability was performed by boiling CFS (pH 6.0) at two different temperatures, 100 and 121°C. At 100°C, the boiling time intervals were 5, 30 and 60 min and at 121°C was 15 min. After heating, antibacterial activity was determined by using spot-on-lawn method. The 10 day-investigation of chill temperature (4°C) on bacteriocin activity was done at 0, 1, 3, 5, 7 and 10 days.

Results

1. Isolation and screening of bacteriocin-producing lactic acid bacteria

Four hundred colonies of LAB were isolated from gastrointestinal tract of 6 fish species; *Lates calcarifer* (seabass; 71 isolates), *Channa stiata* (striped snake-head fish; 48 isolates), *Clarias batrachus* (catfish; 91 isolates), *Barbonymus gonionotus* (javanese barb; 50 isolates), *Orcochromis niloticus* (nile tilapia; 69 isolates) and *Pangasianodon hypophthalmus* (striped catfish; 71 isolates). These isolates were investigated for their antibacterial activity with cell free supernatant (CFS), which eliminated acid condition by adjusting pH 6.0, against 17 bacteria strains. Only one isolate taken from seabass implied to produce bacteriocin and was later designed as Sb2 (Table 1). CFS of Sb2 displayed antibacterial activity against both gram positive and gram negative bacteria as shown in Table 2. The highest antibacterial activity of 12,800 Au/ml was found on

Lb. sakei subsp. *sakei* JCM 1157^T. In addition, CFS of Sb2 exhibited antibacterial activity against both pathogenic bacteria such as *S. aureus* TISTR118 and spoilage bacteria in meat such as *Leuc. mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Br. campestris* NBRC 11547^T, *P. fluorescens* JCM 5963^T, *L. innocua* ATCC 33090^T and *B. coagulans* JCM 2257^T.

Table 1. Isolation of bacteriocin-producing LAB from gastrointestinal tract of 6 fish species

Sources	No. of samples	No. of isolates	No. of bacteriocin-producing LAB	Name
Lates calcarifer (seabass)	6	71	1	Sb2
Channa striata (striped snake-head fish)	4	48	0	-
Clarias batrachus (catfish)	10	91	0	-
Barbonymus gonionotus (javanese barb)	3	50	0	-
Orecochromis niloticus (nile tilapia)	10	69	0	-
Pangasianodon hypophthalmus (striped catfish)	6	71	0	-
Total	39	400	1	

Table 2. Antibacterial activity of isolate Sb2 against bacteria strains

Bacteria strains	Media	Temp.(°C)	Antibacterial activity (AU/ml)
Lactic acid bacteria			
Lactobacillus plantarum ATCC 14917^T	MRS	30	800
Lactobacillus sakei subsp. *sakei* JCM 1157^T	MRS	30	12,800
Lactobacillus sakei TISTR 890	MRS	37	1,600
Lactococcus lactis subsp.*cremoris* TISTR 1344	MRS	30	200
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	MRS	30	800
Leuconostoc mesenteroides subsp. *mesenteroides* TISTR 942	MRS	30	1,600
Enterococcus faecalis JCM 5803^T	MRS	37	800
Enterococcus faecalis TISTR 888	MRS	37	1,600
Streptococcus sp. TISTR 1030	MRS	30	1,600
Other Gram-positive bacteria			
Bacillus coagulans JCM 2257^T	TSB-YE	37	800
Bacillus coagulans TISTR 1447	TSB-YE	37	0
Listeria innocua ATCC 33090^T	TSB-YE	37	1,600
Brochotrix campestris NBRC 11547^T	TSB-YE	26	1,600
Staphylococcus aureus TISTR 118	TSB-YE	37	200
Other Gram-negative bacteria			
Pseudomonas fluorescens JCM 5963^T	TSB-YE	26	200
Pseudomonas fluorescens TISTR 358	TSB-YE	26	200
Aeromonas hydrophila TISTR 1321	NB	30	0

ATCC = American Type Culture Collection , Rockville, Md
JCM = Japanese Culture of Microorganism, Wako, Japan
NBRC = National Institute of Technology and Evaluation (NITE) Biological Resource Center
TISTR = Thailand Institute of Scientific and Technological Research
MRS = de Man, Rogosa and Sharpe
TSB-YE = Tryptic soy broth + 0.6%Yeast extract
NB = Nutrient broth

2. Profile of growth and bacteriocin production of isolate Sb2

The growth condition of isolate Sb2 and bacteriocin production were examined at various temperatures: 30, 37 and 42°C as shown in Figure.1 A, B and C. The cell number and optical density value at 600 nm revealed the same trend of growth. The maximum bacteriocin production (12,800 AU/ml) was observed at 30°C for 18 h. While at 37°C or 42°C bacteriocin productions were lower (6400 and 1600 AU/ml, respectively). Therefore, the optimum growth condition for maximum bacteriocin production of isolate Sb2 was at 30°C for 18 h. (OD value = 0.8).

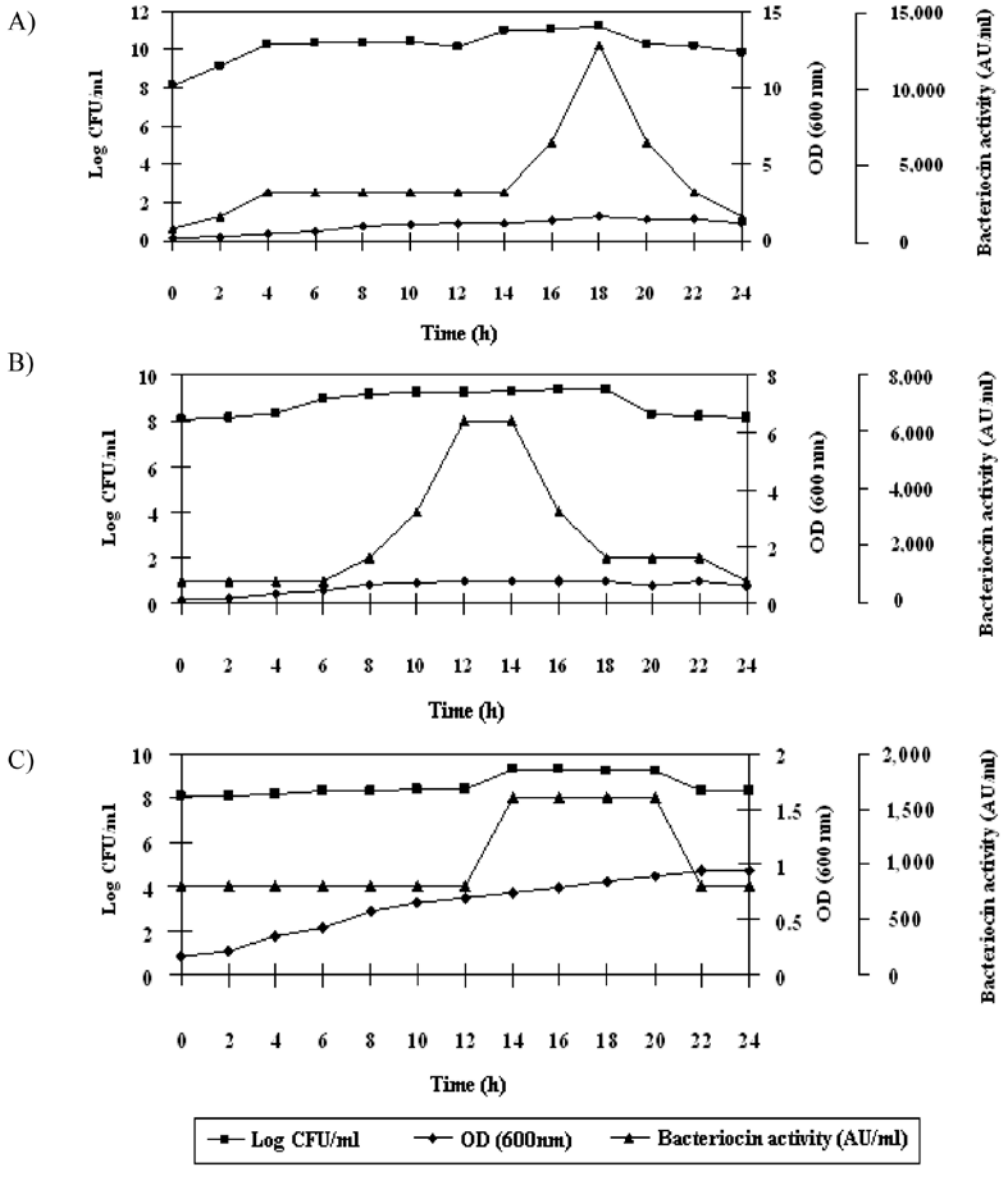

Figure 1. Growth profile of Sb2 and bacteriocin production at various temperatures, A) Profile of growth of Sb2 and bacteriocin production at 30°C, B) Profile of growth of Sb2 and bacteriocin production at 37°C, C) Profile of growth of Sb2 and bacteriocin production at 42°C

3. Effect of proteolytic enzyme

Characterization of CFS by proteolytic enzyme showed a decline of antimicrobial activity when digestion by trypsin, α-chymotrypsin and proteinase K. The remaining antimicrobial activities were 3,200, 400 and 200 AU/ml, respectively, while the control treatment was 6,400 AU/ml, as shown in Table 3.

Table 3. Effect of proteolytic enzyme on the antibacterial activity of Sb2 isolate against *L. sakei* subsp. *sakei* JCM 1157^T

Treatments	Antibacterial activity (Au/ml)
Proteolytic enzyme stability	
Control pH 7	6,400
Trypsin	3,200
α-chymotrypsin	400
Proteinase K	200

4. Effects of heat and chill temperature on bacteriocin activity of CFS

Bacteriocin activity was stabilized for 30 min at 100ºC. At 60 min incubation time, the activity reduced by 50% (from 12,800 to 6,400 AU/ml). In addition, bacteriocin produced from Sb2 could tolerate up to 121ºC for at least 15 min (the activity was reduced from 12,800 to 3,200 Au/ml; as shown in Table 4). The bacteriocin was able to stable in chill temperature (4ºC) up to 5 days (12,800 AU/ml). Its activity gradually decreased to 50% after storing at 4°C for 7 day (6400 AU/ml) and decreased to 25% at day 10 (3,200 Au/ml).

Table 4. Effects of heat and chill temperature on bacteriocin activity of Sb2 against *L. sakei* subsp. *sakei* JCM 1157^T

Treatments	Bacteriocin activity (Au/ml)
Heat Stability	
Control (100˚C 5 min)	12,800
100˚C 10 min	12,800
100˚C 30 min	12,800
100˚C 60 min	6,400
121˚C 15 min	3,200
Chill temperature (4˚C)	
day 0	12,800
1	12,800
3	12,800
5	12,800
7	6,400
10	3,200

Discussion

Even through several studies addressing the bacteriocin-producing LAB isolated from dairy, meat products and fish products, only few bacteriocins isolated from fresh fish have been reported (Campos et al., 2006). The main objectives of this study were to screen and characterization of bacteriocin produced by LAB from gastrointestinal tract of fish. One isolate of a total of 400 LAB was able to produced bacteriocin against *Lb. plantarum* ATCC 14917, *Lb. sakei* subsp. *sakei* JCM 1157^T, *Lb. sakei* TISTR 890, *Lc. lactis* subsp. *cremoris* TISTR 1344, *Leuc. mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Leuc. mesenteroides* TISTR 942, *B. coagulans* JCM 2257^T, *L. innocua* ATCC 33090, *Br. campestris* NBRC 11547, *P. fluorescens* JCM 5693^T, *P. fluorescens* TISTR 358, *E. faecalis* JCM 5803^T, *E. faecalis* TISTR 888, *S. aureus* TISTR 118 and *Streptococcus* sp. TISTR 1030. The maximum bacteriocin production (12,800 AU/ml) was observed at 30°C for 18 hr. Campos et al. (2006) isolated three bacteriocin-producing LAB, (*Lc. lactis* ssp. *lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51). It was found that *Lc. lactis* ssp. *lactis* USC-39 exhibited the maximum antimicrobial activity against *L. monocytogenes* LHICA 1112 after 33 hr at 30ºC of incubation. In addition, antimicrobial activity displayed maximum inhibitory activity against *S. aureus* LHICA 1010 after 21 hr at 30ºC of incubation. While, the highest bacteriocin production by *E. faecium* USC-46 against *L. monocytogenes* LHICA 1112 and *S. aureus* ATCC 35845 was observed after 21 hr at 30ºC. Moreover, bacteriocin production by *E. mundtii* USC-51 showed maximum inhibitory activity against *L. monocytogenes* and *S. aureus* after 46–55 hr and 21 hr at 30ºC of incubation, respectively.

The CFS of Sb2 was found to be sensitive to proteolytic enzymes that indicated the bacteriocin was proteinaceous structure (Klaenhammer, 1988; Vaughan et al., 2001). Its bacteriocin activity was partially inhibited by trypsin, α-chymotrypsin and proteinase K. However, Campos et al. (2006) reported that antimicrobial activities of *Lc. lactis* ssp. *lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 were sensitive to the action of proteinase K. In addition, bacteriocin of Sb2 was able to tolerate when subjected to thermal treatment at 100ºC and chill temperature (4ºC) for 10 days. These experimental results have shown the similar trend to another research done by Campos et al. (2006) who reported that bacteriocin activity of *Lc. lactis* ssp. *lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 was stable in thermal treatment at 100ºC and even stored at 4ºC for 21 days. As a result, bacteriocin produced by Sb2 could be applied to use as biopreservative in food industry.

Conclusion

Bacteriocin-producing lactic acid bacteria Sb2 isolated from seabass gastrointestinal tract. Cell free supernatant (CFS) of Sb2 displayed antibacterial activity against *Lb. plantarum* ATCC 14917^T, *Lb. sakei* subsp. *sakei* JCM 1157^T, *Lb. sakei* TISTR 890, *Lc. lactis* subsp. *cremoris* TISTR 1344, *Leuc. mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Leuc. mesenteroides* subsp. *mesenteroides* TISTR 942, *E. faecalis* TISTR 888, *E. faecalis* JCM 5803^T, *Streptococcus* sp. TISTR 1030, *B. coagulans* JCM 2257^T, *L. innocua* ATCC 33090^T, *Br. campestris* NBRC 11547^T *S. aureus* TISTR 118, *P. fluorescens* JCM 5963^T and *Pseudomonas fluorescens* TISTR 358. The CFS of Sb2 was sensitive to proteolytic enzymes, indicated that bacteriocin was proteinaceous structure. Therefore, bacteriocin produced from Sb2 could be used as biopreservative.

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